

1 **CYTOTOXICITY MEDIATION OF CELLS EVIDENCING SURFACE**

2 **EXPRESSION OF MCSP**

3
4 **Reference to Related Applications:**

5 This application is a continuation-in-part of application S.N. 10/762,129, filed
6 January 20, 2004, which is a continuation-in-part of application S.N. 10/743,451, filed
7 December 19, 2003, which is a continuation of application S.N. 10/348,231, filed January
8 21, 2003, the contents of each of which are herein incorporated by reference.

9 **Field Of The Invention:**

10 This invention relates to the diagnosis and treatment of cancerous diseases,
11 particularly to the mediation of cytotoxicity of tumor cells; and most particularly to the use
12 of cancerous disease modifying antibodies (CDMAB), optionally in combination with one
13 or more chemotherapeutic agents, as a means for initiating the cytotoxic response. The
14 invention further relates to binding assays, which utilize the CDMAB of the instant
15 invention.

16 **Background Of The Invention:**

17 Melanoma-associated chondroitin sulfate proteoglycan (MCSP) was identified
18 independently by several investigators who developed monoclonal antibodies to human
19 metastatic melanoma cell lines. Several antibodies were found to react with a specific
20 antigen associated with the melanoma cell surface. The independent development of these
21 antibodies led to the multiplicity of names for the target antigen, all of which were
22 subsequently determined to be MCSP. MCSP has therefore also been referred to as high
23 molecular weight melanoma associated antigen (HMW-MAA), human melanoma
24 proteoglycan (HMP), melanoma-associated proteoglycan antigen (MPG) and melanoma

1 chondroitin sulfate proteoglycan (mel-CSPG), and has been identified as the antigen of
2 various specific antibodies, some of which have been set out below. MCSP was also found
3 to be over 80 percent homologous with the rat proteoglycan NG2 and is hence also referred
4 to by that name.

5 MCSP is a glycoprotein-proteoglycan complex consisting of an N-linked
6 glycoprotein of 250 kDa and a proteoglycan component >450 kDa. The core glycoprotein
7 is present on the surface of melanoma cells, either as a free glycoprotein or modified by the
8 addition of chondroitin sulfate. The molecular cloning of MCSP led to the identification of
9 several structural features. There are 3 extracellular domains containing a total of 10
10 cysteines (5 potential disulfide bridges), 15 possible N-linked glycosylation sites, and 11
11 potential chondroitin sulfate attachment sites. The transmembrane segment has a single
12 cysteine, however the functional significance of that residue has not been established. The
13 cytoplasmic domain has 3 threonine residues that may serve as sites for phosphorylation by
14 protein kinase C, although it has not yet been shown that MCSP is phosphorylated.

15 It has been shown that MCSP is expressed in the majority of melanoma cancers,
16 and it was originally thought that it had a very limited distribution on normal cells and
17 other tumor types. One early study that led to this conclusion used immunohistochemistry
18 (IHC) on normal and tumor tissues fixed with formaldehyde or methanol in order to
19 determine the distribution of MCSP using anti-MCSP antibody B5. In this study, antibody
20 B5 was found to react with 17 out of 22 melanoma tumors tested, 2 out of 2 astrocytomas
21 tested, and none of the 23 carcinomas tested. Out of 22 normal tissues tested, B5 was
22 found to bind only skin keratinocytes, lung alveolar epithelium and capillary endothelium.

23 Another study examined the tissue distribution of MSCP as defined by anti-MCSP
24 antibody 9.2.27 using frozen tissue sections. Again, reactivity was found in all melanoma

1 tissues and cell lines tested, but there was no reactivity in any of the 6 various carcinoma
2 tumors tested. Out of the 7 fetal tissues tested, reactivity was only observed in the skin and
3 faintly in the aorta while in adult tissues, reactivity was only seen in 3 out of 13 tissues
4 tested.

5 A subsequent study examined the distribution of MCSP using the anti-MCSP
6 antibodies B5, 9.2.27, 225.28S and A0122, all of which recognize distinct epitopes of
7 MCSP. This study was performed on frozen tissues. It was found that all of the anti-
8 MCSP antibodies had similar staining patterns, reacting with normal and malignant tumors
9 of neural, mesenchymal and epithelial origin, that were previously thought to be MCSP
10 negative. Specifically, the antibody B5 reacted with various epithelial, connective, neural
11 and muscular tissues in the 24 organs that were tested, and reacted with 28 out of 34
12 various tumors tested. The authors explained that the differences between their findings
13 and previous reports were due to the use of improved and more consistent IHC techniques,
14 noting that choice of fixative was important, presumably leading to the conclusion that an
15 important characteristic of the MCSP antigen is its sensitivity to the processing steps
16 involved in IHC.

17 A further study was carried out in order to localize MCSP at the ultrastructural
18 level. Immunolocalization studies using electron microscopy demonstrated that MSCP
19 was localized almost exclusively to microspikes, a microdomain of the melanoma cell
20 surface that may play a role in cell-cell contact and cell-substratum adhesion.

21 The molecular cloning of MCSP in 1996 enabled northern blot analysis of MCSP
22 expression in tumor cell lines and normal human tissues using MCSP cDNA probes. Out
23 of 8 various tumor cell lines tested, expression of MCSP was observed only in the
24 melanoma cell line. MCSP expression was not seen in any of the 16 normal adult and 4

1 normal fetal tissues tested. The discrepancies found in different studies of tissue
2 localization of MCSP indicate that further study may be required to elucidate the actual
3 expression patterns of this antigen or to account for the differences that have been reported.

4 Since proteoglycans have been known to mediate cell-cell and cell-extracellular
5 matrix (ECM) interactions, the role of MCSP in these processes has been investigated.
6 MCSP has been shown to stimulate $\alpha_4\beta_1$ -integrin mediated adhesion and spreading of
7 melanoma cells, and it has also been proposed that signaling through the MCSP core
8 protein induces recruitment and tyrosine phosphorylation of p130^{cas} which may regulate
9 cell adhesion and motility, contributing to tumor invasion and metastasis. The
10 combination of these results indicated that MCSP may function to enhance adhesion of
11 melanoma cells by both activating integrins and stimulating pathways that lead to
12 cytoskeletal rearrangement.

13 MCSP has also been found to associate with membrane-type 3 matrix
14 metalloproteinase (MT3-MMP), likely through the chondroitin sulfate component of
15 MCSP. It has been suggested that MT3-MMP expression in melanomas *in vivo* could
16 promote the degradation of ECM proteins in the vicinity of the growing tumor, providing
17 space in which the tumor can expand. Therefore, the association between MT3-MMP and
18 MCSP may be an activation step to promote melanoma invasion.

19 Several *in vitro* assays using anti-MCSP antibodies have been carried out to
20 examine the role of MCSP in processes linked to tumor invasion and metastasis. The role
21 of MCSP in anchorage-independent growth was assessed using the antibody 9.2.27.
22 Melanoma cells cultured in soft agar containing 9.2.27 showed a 67-74 percent specific
23 decrease in their colony formation. These findings suggested that MCSP might be
24 involved in cell-cell interaction, and contribute to anchorage-independent growth. The

1 same authors also examined the effects of blocking MCSP with 9.2.27 in assays measuring
2 the adhesion of M14 melanoma cells on basement membranes of bovine aorta endothelial
3 (BAE) cells. The effect of 9.2.27 treatment was compared to treatment with a control
4 monoclonal antibody W6/32 (directed against all class I histocompatibility antigens). M14
5 control cells and M14 cells pretreated with antibody were plated on basement membranes
6 of BAE cells. A significant inhibition of 27 percent in cell adhesion was observed in
7 9.2.27 treated cells, whereas no significant effect was observed in W6/32 treated cells. A
8 more striking effect of cell pretreatment with 9.2.27 was the inhibition of cell spreading
9 which was verified at the ultrastructural level using scanning electron microscopy.

10 Many of the antibodies that were developed against melanoma cells and determined
11 to specifically recognize MCSP have been tested in both *in vitro* and *in vivo* assays to
12 determine their anti-cancer effects.

13 Monoclonal antibody 9.2.27 recognizes the core glycoprotein component of MCSP
14 and was one of the first antibodies investigated for tumor suppressing properties. Bumol *et*
15 *al.* investigated 9.2.27 and a diphtheria toxin A (DTA) conjugate of 9.2.27 for
16 immunotherapy of melanoma tumors grown in nude mice. *In vitro* cytotoxicity assays
17 were first carried out by measuring the effects of both 9.2.27 and 9.2.27-DTA conjugate on
18 protein synthesis in M21 human melanoma cells as indicated by protein incorporation of
19 [³⁵S]methionine. The 9.2.27-DTA conjugate significantly inhibited protein synthesis in
20 M21 melanoma cells, though a greater effect was seen with unconjugated DTA. There was
21 only minimal effect achieved by 9.2.27 alone. Both the 9.2.27 and 9.2.27-DTA conjugate
22 were investigated for anti-tumor effects in human melanoma tumor-bearing nude mice.
23 M21 tumor mince was implanted subcutaneously and allowed to establish growth for 3
24 days, then mice were treated at day 3 and at 3 day intervals thereafter with either 9.2.27 or
McHale & Slavin, P.A.
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1 9.2.27-DTA conjugate. Tumor volumes of treated mice were compared to those of
2 untreated control mice. At day 18 (the last day for which data was reported), 9.2.27 treated
3 mice showed a 64 percent inhibition of tumor growth while 9.2.27-DTA conjugate treated
4 mice showed a 52 percent inhibition of tumor growth, compared to untreated controls. In
5 this initial study the authors concluded that 9.2.27 and 9.2.27-DTA conjugate were
6 approximately equivalent in their effect on suppression of growth of M21 melanoma
7 tumors in nude mice. While this initial study reports *in vivo* suppression of tumor growth
8 by treatment with 9.2.27, several subsequent studies, including those by the same authors,
9 have demonstrated that naked 9.2.27 did not exhibit any anti-tumor effects *in vivo*.
10 Collectively, as outlined below, the experiments carried out to investigate the utility of
11 using 9.2.27 to treat human tumors have demonstrated that, although cancer cells were
12 targeted by 9.2.27, no anti-cancer activity resulted from treatment with the naked antibody.

13 A phase I clinical trial was carried out which was designed to give large doses of
14 9.2.27 in anticipation of later therapeutic studies with 9.2.27 immunoconjugates. Eight
15 patients with malignant melanoma whose tumors reacted with 9.2.27 by flow cytometry
16 and/or immunoperoxidase staining, received single doses of antibody intravenously, twice
17 weekly on a dose escalating scale of 1, 10, 50, 100 and 200 mg. Although none of the
18 patients experienced significant toxicity and 9.2.27 localized to the metastatic melanoma
19 nodules, no clinical responses were observed.

20 In a later study, 9.2.27 was conjugated to the chemotherapeutic drug doxorubicin
21 (DXR), and the conjugate was investigated for growth inhibition of melanoma *in vitro* and
22 *in vivo*. Growth inhibition of M21 cells treated with the DXR-9.2.27 conjugate was
23 measured using a [³H]thymidine incorporation assay. The conjugate showed specific dose-
24 dependent growth inhibition of the M21 target cells and no effect on an MCSP negative

1 control cell line. No *in vitro* assays were carried out examining effects of 9.2.27 alone. To
2 investigate the DXR-9.2.27 conjugate *in vivo*, M21 cells were injected subcutaneously and
3 allowed to establish a tumor for 8-10 days. Injections were given intravenously at day 10
4 and at 3 day intervals thereafter for 30 days. Significant suppression of tumor growth was
5 seen only in mice treated with the DXR-9.2.27 conjugate. Both DXR treatment alone and
6 9.2.27 treatment alone failed to suppress tumor growth; a mixture of 9.2.27 and DXR
7 showed similar negative effects.

8 Another study was carried out investigating the effects of a 9.2.27 conjugate.
9 Schrappe *et al.* conjugated the chemotherapeutic agent 4-desacetylvinblastine-3-
10 carboxyhydrazide (DAVLBHY) to 9.2.27 and tested its effect on human gliomas. Nude
11 mice were injected with U87MG (a human glioma cell line) cells subcutaneously and the
12 animals were treated on days 2, 5, 7, and 9. Tumor volume was most effectively reduced
13 by the 9.2.27-DAVLBHY conjugate. Control groups, which were treated with either PBS
14 or 9.2.27 alone, developed fast growing tumors and there was no reduction in tumor
15 volume in 9.2.27 treated mice compared to mice treated with PBS.

16 Antibody 225.28S was made against the human M21 melanoma cell line, and was
17 initially described as reacting with a high molecular weight melanoma associated antigen.
18 This molecule was subsequently shown to be the same molecule as MCSP. An early study
19 tested the cytolytic ability of 225.28S, an IgG_{2a}, on a human melanoma cell line and
20 compared it to another anti-MCSP antibody, clone 653.40S that was an IgG₁. 225.28S and
21 653.40S were determined to recognize the same, or spatially close, antigenic determinants
22 on MCSP. It was found that neither antibody could lyse melanoma cells in conjunction
23 with complement in *in vitro* assays. Both antibodies could mediate lysis of target
24 melanoma cells in an antibody-dependent cell-mediated (ADCC) cytotoxicity assay, with

225.28S exhibiting a higher lytic activity than 653.40S. However, lysis of melanoma cells was only obtained with a significantly higher effector/target cell ratio than had been reported by others using anti-melanoma antigen antibodies. The authors concluded that the lack of cytolytic activity of these antibodies in conjunction with human complement and the high effector/target cell ratio required for lysis to occur in ADCC suggested that the injection of monoclonal antibodies into melanoma patients was not likely to cause the destruction of tumor cells. The authors suggested that the immunotherapeutic use of these antibodies should be limited to utilizing them as carriers of radioisotope, chemotherapeutic or toxic agents.

Naked antibody 225.28S was investigated for its therapeutic potential in a phase I trial where it was delivered intravenously in 10 mg doses to 2 patients with end-stage melanoma. Although no clinically adverse or major toxic effects were noted that could be ascribed to the administration of the antibody, there was also no positive therapeutic response.

Antibody 225.28S was conjugated to puromycin, a low molecular weight polypeptide that is especially toxic to dividing cells, and was tested for its *in vitro* toxicity to the human melanoma cell line Colo 38. It was found that the culture of Colo 38 cells with the 225.28S-puromycin conjugate for 24 hr inhibited ³H-thymidine uptake. In addition, the viability of Colo 38 cells was dramatically reduced in cultures incubated with the conjugate for 7 days. Although *in vitro* toxicity was observed, there was still a fraction of melanoma cells that survived the 225.28S-puromycin treatment. The authors suggested that the immunotherapy of malignant diseases may have to rely on cocktails of monoclonal antibodies to distinct tumor associated antigens as carriers of toxic agents, indicating that 225.28S conjugate alone would not be sufficient for treatment of cancer. The effect of

1 225.28S-purothionin conjugate treatment was evaluated on the growth of human
2 melanoma in nude mice. Colo 38 cells were implanted either subcutaneously or
3 intraperitoneally in nude mice. Treatments were made on days 1, 3 and 5 for the
4 intraperitoneal implanted mice and on days 1, 3, 5 and 20 for the subcutaneous implanted
5 mice. Survival was monitored for all mice. The only statistically significant prolongation
6 of survival was observed in the intraperitoneal implanted mice that were treated with the
7 225.28S-purothionin conjugate. 225.28S alone, purothionin alone or a mixture of 225.28S
8 and purothionin did not enhance survival in either the intraperitoneal or the subcutaneous
9 implanted mice. Tumor volume was also recorded for the subcutaneous implanted mice
10 and it was found that only the 225.28S-purothionin conjugate treatment significantly
11 reduced tumor volume. Treatment with 225.28S alone did not result in a reduction of
12 tumor volume.

13 225.28S was also conjugated to the chemotherapeutic drug methotrexate (MTX)
14 and its effects on tumor growth were investigated *in vivo*. Nude mice were inoculated
15 subcutaneously with M21 human melanoma cells and treated on days 1, 4, 7, 10 and 14.
16 The MTX-225.28S conjugate was the only treatment that inhibited tumor growth. 225.28S
17 alone, MTX alone or a mixture of 225.28S and MTX failed to inhibit tumor growth.

18 225.28S was used in a study designed to investigate the potential toxic effects in
19 humans due to the administration of a reagent of a xenogenic nature. 85 patients with
20 metastatic cutaneous melanoma were administered either intact 225.28S or the F(ab')₂
21 fragment that were labeled with ¹³¹I, ¹²³I, ¹¹¹In, or ⁹⁹Tc. The amount of injected antibody
22 ranged from 14 to 750 µg. No clinically detectable side effects were observed in any of the
23 patients. No clinical response was reported, though it was not necessarily anticipated as
24 this study was designed for toxicologic purposes.

1 225.28S was used to generate murine anti-idiotypic monoclonal antibodies
2 including the antibody MF11-30, which bears the mirror image of MCSP. MF11-30 has
3 been shown to induce the development of anti-MCSP antibodies in both a syngeneic and
4 xenogeneic system. MF11-30 was tested in 2 clinical trials in escalating doses designed to
5 test the toxicity and response in patients with advanced malignant melanoma. In both
6 studies there were few side effects due to administration of the antibody and the therapy
7 was well tolerated. In the second trial the average survival of 7 patients who developed
8 anti-anti-idiotypic antibodies with a titer of at least 1:8 and displayed no marked changes
9 in the level of serum MCSP was 55 weeks (range 16-95), which was significantly higher
10 than the remaining 12 patients (who developed anti-antiidiotypic antibodies with a titer of
11 1:4 or less and displayed an increase in the serum level of MCSP) whose average survival
12 was 19 weeks (range 8-57).

13 Antibody 763.74 was also generated against melanoma cells and recognizes MCSP.
14 There have not been any reports of *in vitro* or *in vivo* anti-cancer effects of antibody
15 763.74, however this antibody was also used to generate murine anti-idiotypic monoclonal
16 antibodies. One of these antibodies, MK2-23, bears the internal image of the determinant
17 defined by the anti-MCSP antibody 763.74. In preclinical experiments, immunization with
18 MK2-23 was shown to induce the development of anti-MCSP antibodies in both a
19 syngeneic host (BALB/c mice) and in a xenogenic host (rabbit). The immunogenicity of
20 MK2-23 was markedly enhanced when it was conjugated to the carrier protein keyhole
21 limpet hemocyanin (KLH) and administered with an adjuvant. A clinical trial was carried
22 out to characterize the humoral response induced by MK2-23 in patients with melanoma.
23 25 patients with stage IV melanoma were immunized on days 0, 7 and 28 with 2 mg
24 subcutaneous injections of MK2-23 conjugated to KLH and mixed with Bacillus Calmette

1 Guerin (BCG). Additional injections were given if the titer of anti-anti-idiotypic
2 antibodies was low. Approximately 60 percent of the patients who were immunized with
3 MK2-23 developed anti-MCSP antibodies, although the level and affinity of the anti-
4 MCSP antibodies were low. It was found that survival of patients who developed anti-
5 MCSP antibodies after immunization with MK2-23 was significantly longer than those
6 who did not. The median survival of patients who developed anti-MCSP antibodies was
7 52 weeks (range 19-93) while the median survival of the 9 patients without detectable anti-
8 MCSP antibodies in their sera was 19 weeks (range 9-45). Three patients who developed
9 anti-MCSP antibodies experienced a partial remission of their disease. Although
10 promising results were achieved in this study, 40 percent of the patients immunized with
11 MK2-23 did not respond with detectable anti-MCSP antibodies. As well, the 3 patients
12 who had achieved partial remission all eventually experienced recurrence of disease. An
13 attempt was made to increase the immunogenicity of MK2-23 by pretreatment of patients
14 with a low dose of cyclophosphamide (CTX), which had been reported to enhance the
15 cellular and humoral response to tumor associated antigens by selectively inactivating
16 some sets of suppressor cells. However, no effects of pretreatment with CTX on the
17 immunogenicity of MK2-23 were detected.

18 Monoclonal antibody 48.7 was developed against the human metastatic melanoma
19 cell line M1733 and was reported to react against a molecule subsequently determined to
20 be MCSP. 48.7 was administered in a phase I clinical trial in combination with the murine
21 monoclonal antibody 96.5, which is directed against the transferrin-like cell surface
22 glycoprotein p97 that is present on human melanomas. Five patients received 2 mg each
23 of mAbs 96.5 and 48.7 on day 1, 10 mg each on day 2, and 25 mg each on days 3 through
24 10. Treatment was well tolerated; however there were no clinical responses to treatment

and disease progression occurred in all patients. These two antibodies were investigated in a separate clinical trial where 3 patients with melanoma metastatic to the central nervous system were treated with radiolabeled Fab fragments of either one of these antibodies. Two patients received 5 mg doses of ¹³¹I-labeled Fab fragment of 48.7 in conjunction with osmotic opening of the blood-brain barrier (BBB) in an effort to enhance entry of the antibody into tumors in the brain. The osmotic BBB modification increased the delivery of Fab to the tumor-bearing hemisphere and spinal fluid, but clear persistent localization of the antibody to the tumor was not shown. The authors hypothesized that the lack of localization may have been due to an inadequate dose of the antibody.

Melimmune was a dual preparation of two murine anti-idiotypic antibodies, Melimmune-1 (I-Mel-1) and Melimmune-2 (I-Mel-2), which mimic separate epitopes of MCSP. I-Mel-1 was a subclone of the anti-idiotypic antibody MF11-30, which was developed against the anti-MCSP antibody 225.28 (as previously discussed). I-Mel-1 was shown to induce an anti-MCSP response in rabbits. I-Mel-2 was an anti-idiotypic antibody developed against the anti-MCSP antibody MEM136, which reacts to a different epitope on MCSP than does 225.28. I-Mel-2 was also shown to induce an anti-MCSP response in rabbits. The Melimmune preparation, which contained a 1:1 composition of I-Mel-1 and I-Mel-2, was tested in a phase I trial of 21 patients with resected melanoma without evidence of metastatic disease. Detailed immune response analysis was reported for 12 of these patients enrolled in a single institution. Patients received Melimmune on 1 of 2 treatment schedules with doses that ranged from 0.2 to 4.0 mg (0.1 to 2.0 mg each of I-Mel-1 and I-Mel-2). All patients developed both anti-I-Mel-1 and anti-I-Mel-2 antibodies with the peak antibody response to I-Mel-2 greater than that to I-Mel-1 in 10 out of 12 patients. However, this study was unable to demonstrate induction of specific antibodies to MCSP

1 since none of the patient's sera was able to inhibit either binding of radiolabeled 225.28 to
2 MCSP expressing Mel-21 cells, or binding of radiolabeled MEM136 to Mel-21 cells. A
3 direct cell binding assay was also used to assay for the presence of anti-MCSP antibodies
4 in patients sera; however, there was no difference in the binding of preimmune sera
5 compared to postimmune sera to M21 cells in a FACS based assay.

6 I-Mel-2 was tested in a separate clinical trial where 26 patients with metastatic
7 melanoma were treated with 2 mg I-Mel-2 and either 100 or 250 µg of the adjuvant SAF-m
8 delivered intramuscularly biweekly for 4 weeks and then bimonthly until disease
9 progression. Anti-MCSP antibodies were detected in 5 of the 26 patients using an
10 inhibition radioimmunoassay. Of the 5 patients with detectable anti-MCSP antibodies, 1
11 patient experienced a complete remission, 1 had stable disease and the other 3 had
12 progressive disease. The patient with complete remission had the highest titer of anti-
13 MCSP antibodies (1:1500).

14

15 Prior Patents:

16 US5270202 (and its related patents: WO9216646A1, EP0576570A1) teaches an
17 anti-idiotypic antibody, IMelpg2 (also known as "IM32") to MEM136, an antibody
18 directed to human melanoma-associated proteoglycan (also known as "HMW-MAA"). The
19 IMelpg2 antibody was shown to be directed to MEM136 specifically, and suggested to be
20 of use for the diagnosis and treatment of disease in which cells expressed the MPG
21 epitope. Although there was an effect of IMelpg2 on tumor cell invasion, as determined by
22 *in vitro* assays it was neither the most effective antibody tested, nor was there indications
23 of *in vivo* anti-tumor effects despite showing an Ab3 response.

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2 EP0380607B1 teaches anti-idiotypic antibodies to the Mab 225.28 which has
3 specificity for an undefined epitope of HMW-MAA. These antibodies are useful as active
4 immunotherapy for melanoma. Both MF11-30 and IMelpg1, and polyclonal anti-idiotypic
5 antibodies to 225.28 have been reported and evaluated in animal models with MF11-30
6 undergoing clinical trials in melanoma patients, although there was no supporting data.
7 MF11-30 can induce 225.28 idiotypic antibodies. The IMelpg1 cell line was derived from
8 treating the MF11-30 cell line with BM Cycline and testing for the absence of mycoplasma
9 contamination. Although antibodies to IMelpg1 can be induced in rabbit sera, and be
10 shown to bind to the Colo38 melanoma cell, there was no indication of tumoricidal activity,
11 either *in vitro* or *in vivo*.

12

13 US4879225 teaches the production of antibodies from insoluble immune
14 complexes. In this case rat anti-idiotypic antibodies to Mab 9.2.27, an antibody directed
15 against the HMW-MAA, were generated by immobilizing 9.2.27 on protein A-Sepharose
16 for use as an antigen. Antibodies to melanoma cells were produced using a variety of cell
17 or cell lysate complexes conjugated to Sepharose. Murine monoclonal antibodies that
18 bound to melanoma cells, but not normal T-cells or B-cells were compared to 9.2.27.
19 Those that had similar properties to 9.2.27 were selected for further characterization: NR-
20 ML-02, NR-ML-03, NR-ML-04, NR-ML-05, NR-ML-06. Each of these antibodies were
21 positive in a sandwich ELISA assay using 9.2.27 as the capture antibody and solublized
22 SK MEL-28 melanoma membranes as an antigen source. Further these antibodies were
23 characterized as recognizing melanoma tumor cells, and also reacting with smooth muscle
24 and endothelial cells. An additional 61 anti-proteoglycan antibodies were produced with 10

1 recognizing the same determinant as NR-ML-02/ NR-ML-04, 3 antibodies recognized the
2 same determinant as NR-ML-03 or NR-ML-05; 45 did not recognize the same epitope as
3 determined by the 5 antibodies. In US5084396 these antibodies were radiolabelled and
4 compared with 9.2.27 for tumor uptake in nude mice bearing melanoma xenografts. The
5 tumor uptake was the greatest for NR-ML-05 and NR-ML-02, then 9.2.27, and then NR-
6 ML-02. In neither of these inventions were there indications that these antibodies produced
7 reduction in tumor burden of cancerous disease, nor enhanced survival of mammals having
8 cancerous disease.

9
10 US5034223 teaches a method of enhancing delivery of conjugated antibodies to
11 tissues bearing tumor-associated antigens by pretreating with a non-conjugated blocking
12 antibody. Antibodies to HMW-MAA, 9.2.27 and NR-ML-05, were conjugated to
13 technicium 99 (Tc-99) and were administered in the clinical setting after prior
14 administration of unlabelled Mab NR-2AD, an antibody with an anti-idiotypic specific for
15 only 1 patient's B-cell lymphoma. Since these studies were designed using Tc-99 as a
16 reporter radioisotope, which does not have cytotoxic, or radioablative effects there was no
17 evidence of anti-tumor effects although there was enhanced uptake of the anti-HMW-
18 MAA antibodies through the use of this process.

19
20 US5580774 teaches the construction of a chimeric antibody using the antibody
21 genes that encode Mab 9.2.27. No disclosures regarding the diagnosis or treatment of
22 cancerous disease using the chimeric antibody were made.

23

1 US5493009 and US5780029 teaches the murine anti-idiotypic antibody MK2-23,
2 and its conjugates, directed against an anti-HMW-MAA antibody, 763.74. MK2-23 can
3 bind directly to 763.74 and inhibit 763.74 binding to Colo 38 melanoma cells. Further,
4 Ab3 elicited by MK2-23 can directly bind HMW-MAA and can competitively inhibit
5 763.74 binding to Colo 38 melanoma cells. Active immunotherapy was carried out in a
6 clinical trial in stage IV melanoma patients with MK2-23. In 89 percent of patient's post-
7 immunization sera reacted with Colo 38 melanoma cells, and inhibited binding of 763.74
8 to Colo 38 cells suggesting induction of Ab3 antibodies. In 6 of 15 patients there was a
9 reduction in size of metastatic lesions reported but study details were not furnished. The
10 specificity of the antibodies in patient sera was partially characterized and it is unclear
11 whether Ab3 antibodies, to the extent that they were present, were responsible for any of
12 the clinical response observed, since the 763.74 antibody did not have innate anti-tumor
13 effects. US5866124 teaches the chimeric anti-idiotypic antibody MK2-CH γ 1, and its
14 derivatives, directed against an anti-HMW-MAA antibody, 763.74, derived from MK2-23.

15

16 A number of inventions, such as US5017693, US5707603, US5817774,
17 US6248870, US5112954, US6238667, teach conjugating compounds to antibodies
18 directed against HMW-MAA but fail to disclose their utility in treatment of cancerous
19 disease. Importantly, were these antibodies effective as anti-cancer therapies alone, they
20 would not require a conjugate to impart either cytotoxic or cytostatic effects.

21 These patents and patent applications identify MCSP antigens and related
22 antibodies but fail to disclose the isolated monoclonal antibody of the instant invention, or
23 the utility of the isolated monoclonal antibody of the instant invention.

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Summary Of The Invention:

The instant inventors have previously been awarded U.S. Patent 6,180,357, entitled "Individualized Patient Specific Anti-Cancer Antibodies" directed to a process for selecting individually customized anti-cancer antibodies, which are useful in treating a cancerous disease. For the purpose of this document, the terms "antibody" and "monoclonal antibody" (mAb) may be used interchangeably and refer to intact immunoglobulins produced by hybridomas (e.g. murine or human), immunoconjugates and, as appropriate, immunoglobulin fragments and recombinant proteins derived from said immunoglobulins, such as chimeric and humanized immunoglobulins, F(ab') and F(ab')₂ fragments, single-chain antibodies, recombinant immunoglobulin variable regions (Fv)s, fusion proteins etc. It is well recognized in the art that some amino acid sequence can be varied in a polypeptide without significant effect on the structure or function of the protein. In the molecular rearrangement of antibodies, modifications in the nucleic or amino acid sequence of the backbone region can generally be tolerated. These include, but are not limited to, substitutions (preferred are conservative substitutions), deletions or additions. Furthermore, it is within the purview of this invention to conjugate standard chemotherapeutic modalities, e.g. radionuclides, with the CDMAB of the instant invention, thereby focusing the use of said chemotherapeutics. The CDMAB can also be conjugated to toxins, cytotoxic moieties, enzymes e.g. biotin conjugated enzymes, or hematogenous cells, thereby forming antibody conjugates.

1 This application utilizes the method for producing patient specific anti-cancer
2 antibodies as taught in the '357 patent for isolating hybridoma cell lines which encode for
3 cancerous disease modifying monoclonal antibodies. These antibodies can be made
4 specifically for one tumor and thus make possible the customization of cancer therapy.
5 Within the context of this application, anti-cancer antibodies having either cell-killing
6 (cytotoxic) or cell-growth inhibiting (cytostatic) properties will hereafter be referred to as
7 cytotoxic. These antibodies can be used in aid of staging and diagnosis of a cancer, and
8 can be used to treat tumor metastases.

9 The prospect of individualized anti-cancer treatment will bring about a change in
10 the way a patient is managed. A likely clinical scenario is that a tumor sample is obtained
11 at the time of presentation, and banked. From this sample, the tumor can be typed from a
12 panel of pre-existing cancerous disease modifying antibodies. The patient will be
13 conventionally staged but the available antibodies can be of use in further staging the
14 patient. The patient can be treated immediately with the existing antibodies and/or a panel
15 of antibodies specific to the tumor can be produced either using the methods outlined
16 herein or through the use of phage display libraries in conjunction with the screening
17 methods herein disclosed. All the antibodies generated will be added to the library of anti-
18 cancer antibodies since there is a possibility that other tumors can bear some of the same
19 epitopes as the one that is being treated. The antibodies produced according to this method
20 may be useful to treat cancerous disease in any number of patients who have cancers that
21 bind to these antibodies.

22 Using substantially the process of US 6,180,357, and as disclosed in S.N.
23 10/348,231, the mouse monoclonal antibody 11BD-2E11-2 was obtained following

1 immunization of mice with cells from a patient's breast tumor biopsy. The 11BD-2E11-2
2 antigen was expressed on the cell surface of several human cell lines from different tissue
3 origins. The breast cancer cell line MCF-7 and ovarian cancer cell line OVCAR-3 were
4 susceptible to the cytotoxic effects of 11BD-2E11-2 *in vitro*.

5 The result of 11BD-2E11-2 cytotoxicity against MCF-7 and OVCAR-3 cells in
6 culture was further extended by its anti-tumor activity towards these cancer cells when
7 transplanted into mice (as disclosed in S.N. 10/762,129). Pre-clinical xenograft tumor
8 models are considered valid predictors of therapeutic efficacy.

9 In a preventative *in vivo* model of human breast cancer, 11BD-2E11-2 prevented
10 tumor growth and reduced tumor burden (as disclosed in S.N. 10/762,129). At day 51
11 (soon after last treatment), the mean tumor volume in the 11BD-2E11-2 treated group was
12 20 percent of the isotype control. Monitoring continued past 280 days post-treatment. 40
13 percent of the 11BD-2E11-2 treatment group was still alive at over 7.5 months post-
14 implantation. Conversely, the isotype control group had 100 percent mortality after 6.5
15 months post-treatment. Therefore 11BD-2E11-2 enhanced survival and decreased the
16 tumor burden compared to the control-treated groups in a well-established model of human
17 breast cancer.

18 To determine if 11BD-2E11-2 was efficacious in more than one model of human
19 breast cancer, its anti-tumor activity against MDA-MB-468 (MB-468) cells in an
20 established model of breast cancer was determined. 11BD-2E11-2 reduced tumor growth
21 by 25 percent in comparison to the buffer control. Therefore, 11BD-2E11-2 was effective
22 in preventing tumor growth in an established as well as a preventative breast cancer
23 xenograft model. In addition, 11BD-2E11-2 displayed anti-tumor activity in at least two
24 different models of breast cancer.

1 In addition to the beneficial effects in a model of human breast cancer, 11BD-
2 2E11-2 treatment also had anti-tumor activity against OVCAR-3 cells in a preventative
3 ovarian cancer model (as disclosed in S.N. 10/762,129). In this model, body weight was
4 used a surrogate measure of tumor progression. At day 80 post-implantation (16 days after
5 the end of treatment) the mice in the treated group had 87.6 percent the mean body weight
6 of the control group ($p=0.015$). Thus, 11BD-2E11-2 treatment was efficacious as it
7 delayed tumor progression compared to the buffer control treated group in a well-
8 established model of human ovarian cancer. The anti-tumor activities of 11BD-2E11-2, in
9 several different cancer models, make it an attractive anti-cancer therapeutic agent.

10 To determine if 11BD-2E11-2 was efficacious in more than one model of human
11 ovarian cancer, its anti-tumor activity against ES-2+SEAP cells (ES-2 ovarian cancer cells
12 transfected with human placental secreted alkaline phosphatase (SEAP)) in an established
13 model of ovarian cancer was determined. 11BD-2E11-2 enhanced survival in a cohort of
14 mice in the treatment group in comparison to buffer control. In addition, 1 mouse within
15 the 11BD-2E11-2 treatment group displayed greatly reduced circulating SEAP levels after
16 treatment. Circulating SEAP levels can be used as an indicator of tumor burden. Therefore,
17 11BD-2E11-2 was effective in preventing tumor growth in an established as well as a
18 preventative ovarian cancer xenograft model. In addition, 11BD-2E11-2 displayed anti-
19 tumor activity in two different models of human ovarian cancer.

20 In order to validate the 11BD-2E11-2 epitope as a drug target, the expression of
21 11BD-2E11-2 antigen in frozen normal human tissues was determined. By IHC staining
22 with 11BD-2E11-2, the majority of the tissues failed to express the 11BD-2E11-2 antigen,
23 including the cells of the vital organs, such as the liver, kidney and heart. Albeit, there was

1 staining to the smooth muscle fibers of blood vessels in almost all of the tissues. There was
2 also epithelial staining for some of the tissues.

3 Localization of the 11BD-2E11-2 antigen and its prevalence within breast cancer
4 patients is important in assessing the benefits of 11BD-2E11-2 immunotherapy to patients
5 and designing effective clinical trials. To address 11BD-2E11-2 antigen expression in
6 breast tumors from cancer patients, tumor tissue samples from 8 (7 additional samples
7 were completely detached or not representative of the tumor on the microarray slide)
8 individual breast cancer patients were screened for expression of the 11BD-2E11-2
9 antigen. The results of the study showed that 62 percent of tissue samples positively
10 stained for the 11BD-2E11-2 antigen. Expression of 11BD-2E11-2 within patient samples
11 appeared specific for cancer cells as staining was restricted to malignant cells. In addition,
12 11BD-2E11-2 stained 0 of 3 (2 additional samples again were completely detached from
13 the microarray slide) samples of normal tissue from breast cancer patients. When tumors
14 were analyzed based on their stage, or degree to which the cancer advanced, results did not
15 suggest a trend towards greater positive expression with higher tumor stage for 11BD-
16 2E11-2. However, the result was limited by the small sample size.

17 As outlined herein, additional biochemical data also indicate that the antigen
18 recognized by 11BD-2E11-2 is MCSP. This was supported by studies showing that 11BD-
19 2E11-2 immunoprecipitated protein was recognized by an antibody to the rat homologue
20 of MCSP, and that anti-MCSP immunoprecipitated protein was recognized by 11BD-
21 2E11-2. These IHC and biochemical results demonstrate that 11BD-2E11-2 bound to the
22 MSCP antigen. Thus, the preponderance of evidence showed that 11BD-2E11-2 mediated
23 anti-cancer effects through ligation of an unique epitope present on MCSP.

24 *In toto*, this data demonstrates that the 11BD-2E11-2 antigen is a cancer associated

1 antigen and is expressed in humans, and is a pathologically relevant cancer target. Further,
2 this data also demonstrates the binding of the 11BD-2E11-2 antibody to human cancer
3 tissues, and can be used appropriately for assays that can be diagnostic, predictive of
4 therapy, or prognostic. In addition, the cell localization of this antigen is indicative of the
5 cancer status of the cell due to the lack of expression of the antigen in most non-malignant
6 cells, and this observation permits the use of this antigen, its gene or derivatives, its protein
7 or its variants to be used for assays that can be diagnostic, predictive of therapy, or
8 prognostic.

9 A number of distinct anti-MCSP antibodies have been developed and tested in
10 many *in vitro* and *in vivo* systems. In pre-clinical models, with the exception of one study
11 that was not reproduced, naked anti-MCSP antibodies have been shown to be ineffective in
12 tumor reduction or enhancement of survival in several different melanoma models and one
13 glioma model; other cancer types have not been studied with anti-MCSP antibodies. All
14 trials of naked anti-MCSP antibodies in humans have failed to result in any positive
15 clinical outcomes. Naked 11BD-2E11-2 has been shown to enhance survival and decrease
16 tumor burden in murine models of human breast cancer. 11BD-2E11-2 has also inhibited
17 tumor progression and enhanced survival in murine models of human ovarian cancer.
18 Anti-MCSP antibodies have been conjugated to numerous toxic or chemotherapeutic
19 agents, and these conjugates have demonstrated positive *in vivo* results when tested in
20 murine models of melanoma. There have been no reports of anti-MCSP conjugates tested
21 in humans, so the safety of these conjugates is not known. Delivery of monoclonal
22 antibody alone however has been well tolerated with little, if any associated toxicity.
23 Therefore if treatment of a cancer patient with a naked anti-MCSP antibody could result in
24 a positive clinical outcome, it would be beneficial and an improvement upon what is

1 currently available. Conjugation to a toxic agent is not required for 11BD-2E11-2 to
2 exhibit anti-cancer activity; therefore the specific safety concerns associated with
3 administration of antibody-toxin conjugate are not applicable. Many anti-MCSP
4 antibodies have also been used to generate anti-idiotypic antibodies, which have been
5 tested in both animals and humans. In small non-blinded trials, when the immunization of
6 patients with anti-idiotypic antibodies resulted in a detectable anti-MCSP immune
7 response, there was an increase in median survival of these patients compared to patients
8 who did not develop a specific immune response. In the examples given, targeting MCSP
9 to obtain a positive clinical response may result through the administration of anti-
10 idiotypic antibodies. A problem with this approach is that not all patients who were
11 immunized with the anti-idiotypic antibodies developed an anti-MCSP response.
12 Therefore if an anti-MCSP antibody were available that could result in positive clinical
13 outcomes upon direct administration, this would overcome the problem of relying on a
14 patient's own immune response for producing a clinical benefit. 11BD-2E11-2 is such an
15 antibody as it directly targets MCSP and exhibits anti-cancer effects in pre-clinical
16 xenograft tumor models, which are considered valid predictors of therapeutic efficacy.

17 In all, this invention teaches the use of the 11BD-2E11-2 antigen as a target for a
18 therapeutic agent, that when administered can reduce the tumor burden (thereby delaying
19 disease progression) of a cancer expressing the antigen in a mammal, and can also lead to a
20 prolonged survival of the treated mammal. This invention also teaches the use of a
21 CDMAB (11BD-2E11-2), and its derivatives, to target its antigen to reduce the tumor
22 burden of a cancer expressing the antigen in a mammal, and to prolong the survival of a
23 mammal bearing tumors that express this antigen. Furthermore, this invention also teaches
24 the use of detecting the 11BD-2E11-2 antigen in cancerous cells that can be useful for the
25 diagnosis, prediction of therapy, and

1 prognosis of mammals bearing tumors that express this antigen.

2 If a patient is refractory to the initial course of therapy or metastases develop, the
3 process of generating specific antibodies to the tumor can be repeated for re-treatment.
4 Furthermore, the anti-cancer antibodies can be conjugated to red blood cells obtained from
5 that patient and re-infused for treatment of metastases. There have been few effective
6 treatments for metastatic cancer and metastases usually portend a poor outcome resulting
7 in death. However, metastatic cancers are usually well vascularized and the delivery of
8 anti-cancer antibodies by red blood cells can have the effect of concentrating the antibodies
9 at the site of the tumor. Even prior to metastases, most cancer cells are dependent on the
10 host's blood supply for their survival and anti-cancer antibodies conjugated to red blood
11 cells can be effective against *in situ* tumors as well. Alternatively, the antibodies may be
12 conjugated to other hematogenous cells, e.g. lymphocytes, macrophages, monocytes,
13 natural killer cells, etc.

14 There are five classes of antibodies and each is associated with a function that is
15 conferred by its heavy chain. It is generally thought that cancer cell killing by naked
16 antibodies are mediated either through antibody-dependent cell-mediated cytotoxicity
17 (ADCC) or complement-dependent cytotoxicity (CDC). For example murine IgM and
18 IgG2a antibodies can activate human complement by binding the C-1 component of the
19 complement system thereby activating the classical pathway of complement activation
20 which can lead to tumor lysis. For human antibodies, the most effective complement-
21 activating antibodies are generally IgM and IgG1. Murine antibodies of the IgG2a and
22 IgG3 isotype are effective at recruiting cytotoxic cells that have Fc receptors which will

1 lead to cell killing by monocytes, macrophages, granulocytes and certain lymphocytes.
2 Human antibodies of both the IgG1 and IgG3 isotype mediate ADCC.

3 Another possible mechanism of antibody-mediated cancer killing may be through
4 the use of antibodies that function to catalyze the hydrolysis of various chemical bonds in
5 the cell membrane and its associated glycoproteins or glycolipids, so-called catalytic
6 antibodies.

7 There are two additional mechanisms of antibody-mediated cancer cell killing
8 which are more widely accepted. The first is the use of antibodies as a vaccine to induce
9 the body to produce an immune response against the putative antigen that resides on the
10 cancer cell. The second is the use of antibodies to target growth receptors and interfere
11 with their function or to down regulate that receptor so that its function is effectively lost.

12 The clinical utility of a cancer drug is based on the benefit of the drug under an
13 acceptable risk profile to the patient. In cancer therapy survival has generally been the
14 most sought after benefit, however there are a number of other well-recognized benefits in
15 addition to prolonging life. These other benefits, where treatment does not adversely affect
16 survival, include symptom palliation, protection against adverse events, prolongation in
17 time to recurrence or disease-free survival, and prolongation in time to progression. These
18 criteria are generally accepted and regulatory bodies such as the U.S. Food and Drug
19 Administration (F.D.A.) approve drugs that produce these benefits (Hirschfeld *et al.*
20 Critical Reviews in Oncology/Hematology 42:137-143 2002). In addition to these criteria it
21 is well recognized that there are other endpoints that may presage these types of benefits.
22 In part, the accelerated approval process granted by the U.S. F.D.A. acknowledges that
23 there are surrogates that will likely predict patient benefit. As of year-end (2003), there

1 have been sixteen drugs approved under this process, and of these, four have gone on to
2 full approval, i.e., follow-up studies have demonstrated direct patient benefit as predicted
3 by surrogate endpoints. One important endpoint for determining drug effects in solid
4 tumors is the assessment of tumor burden by measuring response to treatment (Therasse *et*
5 *al.* Journal of the National Cancer Institute 92(3):205-216 2000). The clinical criteria
6 (RECIST criteria) for such evaluation have been promulgated by Response Evaluation
7 Criteria in Solid Tumors Working Group, a group of international experts in cancer. Drugs
8 with a demonstrated effect on tumor burden, as shown by objective responses according to
9 RECIST criteria, in comparison to the appropriate control group tend to, ultimately,
10 produce direct patient benefit. In the pre-clinical setting tumor burden is generally more
11 straightforward to assess and document. In that pre-clinical studies can be translated to the
12 clinical setting, drugs that produce prolonged survival in pre-clinical models have the
13 greatest anticipated clinical utility. Analogous to producing positive responses to clinical
14 treatment, drugs that reduce tumor burden in the pre-clinical setting may also have
15 significant direct impact on the disease. Although prolongation of survival is the most
16 sought after clinical outcome from cancer drug treatment, there are other benefits that have
17 clinical utility and it is clear that tumor burden reduction, which may correlate to a delay in
18 disease progression, extended survival or both, can also lead to direct benefits and have
19 clinical impact (Eckhardt et al. Developmental Therapeutics: Successes and Failures of
20 Clinical Trial Designs of Targeted Compounds; ASCO Educational Book, 39th Annual
21 Meeting, 2003, pages 209-219).

22 Accordingly, it is an objective of the invention to utilize a method for producing
23 cancerous disease modifying antibodies from cells derived from a particular individual
24 which are cytotoxic with respect to cancer cells while simultaneously being relatively non-
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1 toxic to non-cancerous cells, in order to isolate hybridoma cell lines and the corresponding
2 isolated monoclonal antibodies and antigen binding fragments thereof for which said
3 hybridoma cell lines are encoded.

4 It is an additional objective of the invention to teach CDMAB and antigen binding
5 fragments thereof.

6 It is a further objective of the instant invention to produce CDMAB whose
7 cytotoxicity is mediated through ADCC.

8 It is yet an additional objective of the instant invention to produce CDMAB whose
9 cytotoxicity is mediated through CDC.

10 It is still a further objective of the instant invention to produce CDMAB whose
11 cytotoxicity is a function of their ability to catalyze hydrolysis of cellular chemical bonds.

12 A still further objective of the instant invention is to produce CDMAB which are
13 useful in a binding assay for diagnosis, prognosis, and monitoring of cancer.

14 Other objects and advantages of this invention will become apparent from the
15 following description wherein, by way of illustration and example, certain embodiments of
16 this invention are set forth.

17

18 Brief Description of the Figures:

19 The patent or application file contains at least one drawing executed in color. Copies of
20 this patent or patent application publication with color drawing(s) will be provided by the
21 Office upon request and payment of the necessary fee.

1 Figure 1. Western blot of MDA-MB-231 (Lane 1) or OVCAR-3 (Lane 2) membranes
 2 probed with 11BD-2E11-2. Membrane proteins were separated under reducing conditions.
 3 Molecular weight markers are indicated on the right.

4 Figure 2. Effect of deglycosylation on the binding of 11BD-2E11-2 to MDA-MB-231
 5 membranes. 11BD-2E11-2 binding to MDA-MB-231 membranes that were incubated in
 6 deglycosylation buffer only (Lane 1), in a combination of PNGase F, endo-o-glycosidase,
 7 sialidase, galactosidase and glucosaminidase (Lane 2), in a combination of PNGase, endo-
 8 o-glycosidase and sialidase (Lane 3), in sialidase only (Lane 4), in endo-o-glycosidase only
 9 (Lane 5), and in PNGase only (Lane 6).

10 Figure 3. SDS-PAGE (Panel A) and Western blot (Panel B) of MDA-MB-231 membrane
 11 proteins immunoprecipitated with 11BD-2E11-2. Lane 1 represents the molecular weight
 12 standard, Lane 2 the MDA-MB-231 membrane proteins, Lane 3 the 11BD-2E11-2
 13 immunoprecipitated material and Lane 4 the isotype control immunoprecipitated material.

14 Figure 4. Western blots of proteins probed with 11BD-2E11-2 (Panel A), IgG1 isotype
 15 control (clone 107.3, Panel B), anti-rat NG2 (polyclonal, Panel C), normal rabbit IgG
 16 (Panel D), anti-MCSP (clone 9.2.27, Panel E) and IgG2a isotype control (clone G155-228,
 17 Panel F). Lane 1: 11BD-2E11-2 immunoprecipitate, Lane 2: IgG1 isotype control (clone
 18 107.3) immunoprecipitate, Lane 3: anti-MCSP (clone 9.2.27) immunoprecipitate, Lane 4:
 19 IgG2a isotype control (clone G155-228) immunoprecipitate, Lane 5: MDA-MB-231
 20 membranes and Lane 6: sample buffer only (negative control).

21 Figure 5. Representative FACS histograms of 11BD-2E11-2, isotype control or anti-
 22 EGFR directed against several cancer cell lines and non-cancer cells.

23 Figure 6. Representative micrographs showing the binding pattern obtained with 11BD-
 24 2E11-2 (A) and the isotype control antibody (B) on tissues sections of heart from a frozen

1 normal human tissue array. There is no staining of 11BD-2E11-2 to cardiac muscle fibers.

2 Magnification is 200X.

3 Figure 7. Representative micrographs showing the binding pattern obtained with 11BD-

4 2E11-2 (A), anti-actin (B) and the isotype control antibody (C) on tissues sections of

5 skeletal muscle from a frozen normal human tissue array. 11BD-2E11-2 did not stain

6 skeletal muscle but there is staining to the smooth muscles of blood vessels (arrow).

7 Magnification is 200X.

8 Figure 8. Representative micrograph of 11BD-2E11-2 (A) and isotype control antibody (B)

9 binding to breast cancer tumor (infiltrating duct carcinoma). The black arrow in panel A

10 points to tumor cells. Magnification is 200X.

11 Figure 9. Effect of 11BD-2E11-2 or buffer control on tumor growth in a preventative

12 MDA-MB-468 breast cancer model. The dashed line indicates the period during which the

13 antibody was administered. Data points represent the mean +/- SEM.

14 Figure 10. Survival of tumor-bearing mice after treatment with 11BD-2E11-2 or buffer

15 control antibody in an established ES-2 xenograft study.

16 Figure 11. SEAP levels of tumor-bearing mice before, during and after treatment with

17 11BD-2E11-2 or buffer control in an established ES-2 xenograft study.

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1 Detailed Description Of The Invention:

2 Example 1

3 **Identification of Binding Proteins by Western Blotting**

4 To identify the antigen(s) recognized by the antibody 11BD-2E11-2, cell
5 membranes expressing this antigen were subjected to gel electrophoresis and transferred
6 using Western blotting to membranes to determine the proteins detected by this antibody.

7

8 **1. Membrane Preparation**

9 Previous work demonstrated binding by FACS of 11BD-2E11-2 to the breast
10 cancer line MDA-MB-231 (MB-231). Previous work also demonstrated 11BD-2E11-2
11 efficacy against the ovarian cancer cell line OVCAR-3. Accordingly, membrane
12 preparations from these 2 cell lines were used for antigen identification. Total cell
13 membranes were prepared from confluent cultures of MB-231 breast cancer or OVCAR-3
14 ovarian cells. Media was removed from cell stacks and the cells were washed with
15 phosphate buffered saline. Cells were dissociated with dissociation buffer (Gibco-BRL,
16 Grand Island, NY) for 20 min at 37°C on a platform shaker. Cells were collected and
17 centrifuged at 900g for 10 min at 4°C. After centrifugation, cell pellets were resuspended
18 in PBS and centrifuged again at 900g for 10 min at 4°C to wash. Pellets were stored at –
19 80°C. Cell pellets were resuspended in homogenization buffer containing 1 tablet per 50
20 mL of Complete protease inhibitor cocktail (Roche, Laval QC) at a ratio of 3 mL buffer
21 per gram of cells. The cell suspension was subjected to homogenization using a polytron
22 homogenizer on ice in order to lyse the cells. The cell homogenate was centrifuged at
23 15,000g for 10 min at 4°C to remove the nuclear particulate. Supernatant was harvested,

1 divided into tubes and then centrifuged at 75,600g for 90 min at 4°C. Supernatant was
2 carefully removed from the tubes and each membrane pellet was resuspended in
3 approximately 5 mL homogenization buffer. The resuspended pellets from all tubes were
4 combined together in one tube and centrifuged at 75,600g for 90 min at 4°C. Supernatant
5 from the tubes was carefully removed, and the pellets were weighed. Solubilization buffer
6 containing 1 percent Triton X-100 was added to the pellets at a ratio of 3 mL buffer per
7 gram of membrane pellet. Membranes were solubilized by shaking on a platform shaker at
8 300 rpm for 1 hr on ice. The membrane solution was centrifuged at 75,600g to pellet
9 insoluble material. The supernatant containing the solubilized membrane proteins was
10 carefully removed from tubes, assayed for protein content, and stored at -80°C.

11

12 **2. SDS-PAGE and Western blot**

13 Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis. 20
14 µg of membrane protein was mixed with SDS-PAGE sample buffer containing 100 mM
15 DTT and was loaded onto a lane of an 8 percent SDS-PAGE gel. A sample of prestained
16 molecular weight markers (Invitrogen, Burlington, ON) was run in a reference lane.
17 Electrophoresis was carried out at 100 V for 10 minutes, followed by 150 V until sufficient
18 resolution of the prestained molecular weight markers was observed. Proteins were
19 transferred from the gel to PVDF membranes (Millipore, Billerica, MA) by electroblotting
20 for 16 hr at 40 V. Transfer was assessed by noting complete transfer of the prestained
21 markers from the gel to the membrane. Following transfer, membranes were blocked with
22 5 percent skim milk powder in Tris-buffered saline containing 0.5percent Tween-20
23 (TBST) for 2 hr. Membranes were washed once with TBST and then incubated with 5

1 $\mu\text{g/mL}$ 11BD-2E11-2 diluted in 3 percent skim milk powder in TBST for 2 hr. After
2 washing 3 times with TBST, membranes were incubated with goat anti-mouse IgG (Fc)
3 conjugated to horseradish peroxidase (HRP) from Jackson Immunologicals (West Grove
4 PA). This incubation was followed by washing 3 times with TBST, followed by
5 incubation with the HRP substrate 3,3',5,5'-tetramethyl benzidine (TMB) (substrate kit
6 from Vector Laboratories, Burlington ON).

7 In Figure 1 11BD-2E11-2 clearly binds to 3 molecular weight regions of the
8 separated MB-231 (Lane 1) and OVCAR-3 (Lane 2) membrane proteins. By comparison
9 to the molecular weight (MW) standards, the antibody binds to proteins of MW
10 approximately 150, 240 and 280 kDa. All further studies were done using the MB-231
11 membranes since stronger reactivity was seen with this cell line.

12 Example 2

13 **Determining Glycosylation of Antigens Bound by 11BD-2E11-2**

14 In order to determine if the antigen(s) recognized by the antibody 11BD-2E11-2
15 were glycoproteins, MB-231 membranes were incubated with different combinations of
16 PNGase F, endo-o-glycosidase, sialidase, galactosidase and glucosaminidase. Membranes
17 were separated by SDS-PAGE followed by Western blotting as described with 11BD-
18 2E11-2. Figure 2 demonstrates the result of 11BD-2E11-2 binding to MB-231 membranes
19 that were incubated in deglycosylation buffer only (Lane 1), in a combination of PNGase
20 F, endo-o-glycosidase, sialidase, galactosidase and glucosaminodase (Lane 2), in a
21 combination of PNGase, endo-o-glycosidase and sialidase (Lane 3), in sialidase only (Lane
22 4), in endo-o-glycosidase only (Lane 5), and in PNGase only (Lane 6). Treatment of MB-
23 231 membranes with glycosidases does not eliminate binding of 11BD-2E11-2, however a

1 molecular weight shift of the proteins is observed in all lanes, indicating that the antigen
2 recognized by 11BD-2E11-2 was a glycoprotein.

3 Example 3

4 **Identification of Antigens Bound by 11BD-2E11-2**

5 **1. Immunoprecipitation**

6 The identification of the antigen for 11BD-2E11-2 was carried out by isolating the
7 cognate ligand through immunoprecipitation of solublized membrane glycoproteins with
8 the antibody. 100 μ L of Protein G Dynabeads (DynaL Biotech, Lake Success NY) were
9 washed 3 times with 1 mL of 0.1 M sodium phosphate buffer pH 6.0. 100 μ g of 11BD-
10 2E11-2 in a total volume of 100 μ L 0.1 M sodium phosphate buffer pH 6.0 was added to
11 the washed beads. The mixture was incubated for 1 hr with rotational mixing. Unbound
12 antibody was removed and the 11BD-2E11-2 coated beads were washed 3 times with 0.5
13 mL 0.1 M sodium phosphate pH 7.4 containing 0.1 percent Tween-20. The 11BD-2E11-2
14 coated beads were washed 2 times with 1 mL 0.2 M triethanolamine pH 8.2. 11BD-2E11-
15 2 was chemically crosslinked to the beads by adding 1 mL of 0.02 M
16 dimethylpimelimidate in 0.2 M triethanolamine pH 8.2 and incubating with rotational
17 mixing for 30 min. The reaction was stopped by incubating the beads with 1 mL of 0.05
18 M Tris pH 7.5, for 15 min with rotational mixing. The 11BD-2E11-2 crosslinked beads
19 were washed 3 times with 1 mL of 1 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 137 mM NaCl, 2.7
20 mM KCl (PBS) containing 0.1 percent Tween-20. The 11BD-2E11-2 crosslinked beads
21 were pre-eluted by incubation with 0.1 M citrate pH 3.0 for 3 min followed by 3 washes in
22 0.1 M PBS containing 0.1 percent Tween-20. A second set of antibody crosslinked beads
23 were prepared in the same manner described using a mouse IgG₁ antibody (clone 107.3

1 from BD Biosciences, Oakville ON) to trinitrophenol, an irrelevant molecule, which was
2 used as a negative IgG₁ isotype control.

3 The 11BD-2E11-2 crosslinked beads were blocked by incubating in 1 percent BSA in
4 0.1 M sodium phosphate pH 7.4 with rotational mixing for 30 minutes at 4°C. The beads
5 were washed 3 times with 0.1 M sodium phosphate pH 7.4. 500 µg of total membrane
6 preparation from MB-231 cells was incubated with the 11BD-2E11-2 crosslinked beads
7 with rotational mixing for 2.5 hr at 4°C. The immunocomplex bound beads were washed
8 three times with 1 mL of 1 mM KH₂PO₄, 10 mM Na₂HPO₄, 287 mM NaCl, 2.7 mM KCl
9 containing 1 percent Triton X-100. 11BD-2E11-2 bound protein was eluted from the
10 11BD-2E11-2 crosslinked beads by incubation with 30 µL of 0.1 M citrate pH 3.0 for 3
11 min with gentle mixing. The eluted protein was brought to neutral pH by the addition of 9
12 µL of 1M Tris pH 9. The neutralized eluted protein was stored at -80 °C. The 11BD-
13 2E11-2 crosslinked beads were washed with 3 mL PBS containing 0.1 percent Tween-20.
14 The IgG₁ isotype control (clone 107.3) crosslinked beads were incubated with MB-231
15 membrane proteins and processed in the same manner as the 11BD-2E11-2 beads.

16 Two batches of 11BD-2E11-2 immunoprecipitated protein from MB-231 membrane
17 proteins were produced as described and combined together. The same was done for the
18 IgG₁ (clone 107.3) isotype control beads. Sixty-two percent of this immunoprecipitate
19 mixture (corresponding to the amount of protein immunoprecipitated from 620 µg of MB-
20 231 membrane proteins) was loaded onto a single lane of a 4-20 percent gradient SDS-
21 PAGE gel. The same amount of material produced from the 107.3 crosslinked beads was
22 loaded in an adjacent lane, as was 20 µg of MB-231 membrane proteins. A sample of
23 unstained molecular weight markers (Invitrogen, Burlington ON) or pre-stained molecular

1 weight markers were run in reference lanes. The sample was separated by electrophoresis
2 at 100 V for 10 min, followed by 150 V for 60 minutes. Proteins were stained by
3 incubating the gel in SYPRO Ruby™ (BioRad, Mississauga, ON). In a parallel Western
4 blot, 18 percent of the immunoprecipitate mixture, which corresponded to the amount of
5 protein immunoprecipitated from 180 µg of MB-231 membrane proteins, and the same
6 amount of material produced from the IgG1 isotype control (clone 107.3) crosslinked
7 beads, were separated by electrophoresis. Proteins were transferred from the gel to PVDF
8 membranes (Millipore, Billerica, MA) by electroblotting for 16 hr at 40 V. After transfer,
9 the membrane was blocked with 5 percent skim milk powder in TBST for 2 hr. The
10 membrane was probed with 5 µg/mL 11BD-2E11-2 diluted in 3 percent skim milk powder
11 in TBST for 2 hr. After washing 3 times with TBST, the membrane was incubated with
12 goat anti-mouse IgG (Fc) conjugated HRP for 1 hr. This incubation was followed by
13 washing 3 times with TBST, followed by incubation with the HRP substrate TMB.

14 Figure 3 depicts the gel and Western blot obtained from the proteins
15 immunoprecipitated by 11BD-2E11-2. On the gel (Panel A) Lane 1 represents the
16 molecular weight standard and Lane 2 represents the MB-231 membrane proteins. There
17 were two distinct bands of MW 240 and 280 kDa in the lane containing the 11BD-2E11-2
18 immunoprecipitated material (Lane 3) that were not present in the lane containing the
19 107.3 immunoprecipitated material (Lane 4). On the corresponding Western blot (Panel
20 B), 11BD-2E11-2 reacts strongly with the 11BD-2E11-2 immunoprecipitated proteins of
21 MW 240 and 280 kDa (Lane 3). On the Western blot 11BD-2E11-2 also reacts strongly to
22 an additional band in the 11BD-2E11-2 immunoprecipitated protein at 150 kDa; this band
23 was not detectable on the stained gel. The reactivity profile of 11BD-2E11-2 to 11BD-
24 2E11-2 immunoprecipitated protein was similar to that seen in the MB-231 total
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1 membranes (Lane 2). There was no reactivity of 11BD-2E11-2 to proteins
2 immunoprecipitated by IgG1 isotype control (clone 107.3; Lane 4), indicating that the
3 binding of 11BD-2E11-2 to the immunoprecipitated protein was specific, and not due to
4 the presence of contaminating proteins.

5 **2. Mass Spectrometry**

6 The regions of the gel corresponding to the 240 and 280 kDa protein
7 immunoprecipitated by 11BD-2E11-2 (Figure 3, Panel A, Lane 3) were cut out using
8 sterile scalpels. These gel slices were then used for identification of proteins by mass
9 spectrometry using MALDI/MS and LC/MS/MS.

10 The samples were subjected to proteolytic digestion on a ProGest workstation using
11 trypsin, and a portion of the resulting digest supernatant was used for MALDI/MS
12 analysis. Spotting was performed robotically (ProMS) with ZipTips; peptides were eluted
13 from the C18 material with matrix (α -cyano 4-hydroxy cinnamic acid) prepared in 60
14 percent acetonitrile, 0.2 percent TFA. MALDI/MS data was acquired on an Voyager DE-
15 STR instrument (Applied Biosystems, Foster City CA and the observed m/z values were
16 submitted to ProFound (Proteometrics software package) for peptide mass fingerprint
17 searching. ProFound queried a locally stored copy of the NCBI nr database. An additional
18 portion of the digest supernatant was analyzed by nano LC/MS/MS on a Micromass Q-
19 ToF2 using a 75 μ m C18 column at a flow-rate of 200 nL/min. MS/MS data were searched
20 using a local copy of MASCOT.

21

22

23

1

2 The proteins identified by MALDI/MS and LC/MS/MS are presented in Table 1.

3

4 Table 1. Proteins Identified by 11BD-2E11-2 Immunoprecipitation of MDA-MB-231
5 Membranes

Sample	Observed MW	Method	Protein ID	Percent coverage	# of peptides matched	NCBI accession #
A	280 kDa	MALDI	Melanoma-associated chondroitin sulfate proteoglycan	13	20	gi 4503099
		LC/MS/MS	Melanoma chondroitin sulfate proteoglycan		2	gi 34148711
B	240 kDa	MALDI	Melanoma associated chondroitin sulfate proteoglycan	14	21	gi 4503099

6

7 Both samples were identified as melanoma-associated chondroitin sulfate proteoglycan
8 (MCSP).

9

10 **3. Confirmation**

11 Confirmation of the putative antigen was carried out by determining whether known
 12 anti-MCSP antibodies would react with the protein immunoprecipitated by 11BD-2E11-2
 13 and vice versa. Immunoprecipitates were prepared in the same manner as described
 14 previously except with the addition of the mouse anti-MCSP monoclonal antibody 9.2.27
 15 (IgG2a) (Chemicon, Temecula CA) and the mouse IgG2a antibody (clone G155-178 from
 16 BD Biosciences; Oakville ON) to trinitrophenol, an irrelevant molecule, which was used as
 17 a negative IgG2a isotype control. 11BD-2E11-2 immunoprecipitate, IgG1 isotype control

(clone 107.3) immunoprecipitate, anti-MCSP (clone 9.2.27) immunoprecipitate, IgG2a isotype control (clone G155-228) immunoprecipitate and MB-231 membranes were separated by SDS-PAGE on six replicate 10 percent gels. Electrophoresis and Western blotting were carried out as described above. The membranes were incubated with 5 µg/mL of 11BD-2E11-2, IgG1 isotype control (clone 107.3), anti-MCSP (clone 9.2.27), IgG2a isotype control (clone G155-228), rabbit polyclonal anti-rat NG2 antibody (MCSP is the human homologue of rat NG2; Chemicon, Temecula CA) and normal rabbit IgG (Sigma, Saint Louis MO) diluted in 3 percent skim milk powder in TBST for 2.5 hr. Figure 4 demonstrates the results of the Western blotting as described. Figure 4 (Panel A) shows the binding of 11BD-2E11-2 to 11BD-2E11-2 immunoprecipitate (Lane 1), IgG1 isotype control (clone 107.3) immunoprecipitate (Lane 2), anti-MCSP (clone 9.2.27) immunoprecipitate (Lane 3), IgG2a isotype control (clone G155-228) immunoprecipitate (Lane 4), MB-231 membranes (Lane 5) and sample buffer only (negative control) (Lane 6). 11BD-2E11-2 recognized the same three bands of approximately 150, 240 and 280 kDa in both the MB-231 membranes and in the 11BD-2E11-2 immunoprecipitate. Only the upper 280 kDa band was recognized in the anti-MCSP (clone 9.2.27) immunoprecipitate lane. There is no reaction in either of the isotype control immunoprecipitate lanes, indicating that the reactivity of 11BD-2E11-2 to the immunoprecipitates was due to proteins being specifically bound and immunoprecipitated by both 11BD-2E11-2 and 9.2.27. In a parallel blot (Panel B) probed with IgG1 isotype control (clone 107.3), no reactivity was observed in any of the lanes, indicating that the reactivity observed in the blot probed with 11BD-2E11-2 was specific. Panel C shows the binding of rabbit polyclonal anti-rat NG2 antibody to a parallel blot. Anti-NG2 binds to two bands of approximately 150 and 240 kDa in the 11BD-2E11-2 immunoprecipitate (Lane 1) while it

1 does not bind to proteins of this molecular weight range in any of the other lanes. In a
2 parallel blot (Panel D), normal rabbit IgG shows faint non-specific reactivity to proteins in
3 both the IgG2a immunoprecipitate (Lane 4) and MB-231 membranes (Lane 5). Therefore
4 the same reactivity in these lanes on Panel C (probed with rabbit anti-NG2) should be
5 regarded as non-specific. In a parallel blot (Panel E) anti-MCSP (clone 9.2.27) shows only
6 very faint binding to one band in the anti-MCSP (clone 9.2.27) immunoprecipitate lane
7 (Lane 3, indicated by arrow); this band is not seen in the MB-231 membranes (Lane 5)
8 which indicates that 9.2.27 may have a low affinity for this antigen and only show
9 reactivity when it is present in a concentrated form such as it is in the immunoprecipitated
10 sample. In the final parallel blot (Panel F) probed with IgG2a isotype control (clone G155-
11 228), no reactivity was observed in any of the lanes, indicating that the reactivity observed
12 in the blot probed with anti-MCSP (clone 9.2.27) was specific. These results demonstrate
13 that 11BD-2E11-2 immunoprecipitated protein was recognized by the rat homologue of
14 MCSP, and that anti-MCSP immunoprecipitated protein was recognized by 11BD-2E11-2.

15 The mass spectroscopic identification combined with the confirmation using known
16 commercial antibodies demonstrates that the antigen for 11BD-2E11-2 is MCSP. This is
17 also consistent with the deglycosylation experiments in Example 2, as the core protein of
18 MCSP is a glycoprotein.

19

20 Example 4

21 As outlined in S.N. 10/743,451, the hybridoma cell line 11BD-2E11-2 was
22 deposited, in accordance with the Budapest Treaty, with the American Type Culture
23 Collection, 10801 University Blvd., Manassas, VA 20110-2209 on November 11, 2003,
24 under Accession Number PTA-5643. In accordance with CFR 1.808, the depositors assure
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1 that all restrictions imposed on the availability to the public of the deposited materials will
2 be irrevocably removed upon the granting of a patent.

3 4 Antibody Production:

5 11BD-2E11-2 monoclonal antibody was produced by culturing the hybridoma in
6 CL-1000 flasks (BD Biosciences, Oakville, ON) with collections and reseeded occurring
7 twice/week. The antibody was purified according to standard antibody purification
8 procedures with Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Baie d'Urfé,
9 QC).

10 As previously described in S.N. 10/348,231, 11BD-2E11-2 was compared to a
11 number of both positive (anti-Fas (EOS9.1, IgM, kappa, 20 micrograms/mL, eBioscience,
12 San Diego, CA), anti-Her2/neu (IgG1, kappa, 10 microgram/mL, Inter Medico, Markham,
13 ON), anti-EGFR (C225, IgG1, kappa, 5 microgram/mL, Cedarlane, Hornby, ON),
14 Cycloheximide (100 micromolar, Sigma, Oakville, ON), NaN₃ (0.1%, Sigma, Oakville,
15 ON)) and negative (107.3 (anti-TNP, IgG1, kappa, 20 microgram/mL, BD Biosciences,
16 Oakville, ON), G155-178 (anti-TNP, IgG2a, kappa, 20 microgram/mL, BD Biosciences,
17 Oakville, ON), MPC-11 (antigenic specificity unknown, IgG2b, kappa, 20
18 microgram/mL), J606 (anti-fructosan, IgG3, kappa, 20 microgram/mL), IgG Buffer (2%))
19 controls in a cytotoxicity assay (Table 2). Breast cancer (MDA-MB-231 (MB-231),
20 MDA-MB-468 (MB-468), MCF-7), colon cancer (HT-29, SW1116, SW620), lung cancer
21 (NCI H460), ovarian cancer (OVCAR-3 (OVCAR)), prostate cancer (PC-3), and non-
22 cancer (CCD 27sk, Hs888 Lu) cell lines were tested (all from the ATCC, Manassas, VA).
23 The Live/Dead cytotoxicity assay was obtained from Molecular Probes (Eugene,OR). The
24 assays were performed according to the manufacturer's instructions with the changes

1 outlined below. Cells were plated before the assay at the predetermined appropriate
2 density. After 2 days, purified antibody or controls were diluted into media, and then 100
3 microliters were transferred to the cell plates and incubated in a 5 percent CO₂ incubator
4 for 5 days. The plate was then emptied by inverting and blotted dry. Room temperature
5 DPBS containing MgCl₂ and CaCl₂ was dispensed into each well from a multi-channel
6 squeeze bottle, tapped three times, emptied by inversion and then blotted dry. 50
7 microliters of the fluorescent calcein dye diluted in DPBS containing MgCl₂ and CaCl₂
8 was added to each well and incubated at 37°C in a 5 percent CO₂ incubator for 30 minutes.
9 The plates were read in a Perkin-Elmer HTS7000 fluorescence plate reader and the data
10 was analyzed in Microsoft Excel and the results were tabulated in Table 1. The data
11 represented an average of four experiments tested in triplicate and presented qualitatively
12 in the following fashion: 4/4 experiments greater than threshold cytotoxicity (+++), 3/4
13 experiments greater than threshold cytotoxicity (++), 2/4 experiments greater than
14 threshold cytotoxicity (+). Unmarked cells in Table 1 represent inconsistent or effects less
15 than the threshold cytotoxicity. 11BD-2E11-2 was specifically cytotoxic in breast and
16 ovarian cancer cells, and did not affect normal cells. The chemical cytotoxic agents
17 induced their expected cytotoxicity while a number of other antibodies which were
18 included for comparison also performed as expected given the limitations of biological cell
19 assays. *In toto*, it was shown that the 11BD-2E11-2 antibody has cytotoxic activity against
20 two cancer cell types. The antibody was selective in its activity since not all cancer cell
21 types were susceptible. Furthermore, the antibody demonstrated functional specificity
22 since it did not produce cytotoxicity against non-cancer cell types, which is an important
23 factor in a therapeutic situation.

24

Table 2		BREAST			COLON			LUNG	OVARY	PROSTATE	NORMAL	
		MB-231	MB-468	MCF-7	HT-29	SW1116	SW620	NCI H460	OVCAR	PC-3	CCD 27sk	Hs88
	11BD-2E11-2	-	-	+	-	-	-	-	+	-	-	-
Positive Controls	anti-Fas	-	-	+++	-	-	-	-	+++	+	-	+
	anti-Her2	+	-	+	-	-	-	-	+	-	-	-
	anti-EGFR	-	+++	+	-	+++	-	-	+	-	+	-
	CHX (100 μ M)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
	NaN ₃ (0.1%)	+++	+++	+++	+++	-	-	+++	+++	+++	-	-
Negative Controls	IgG1							+++		+		
	IgG2a			+++		+						
	IgG2b			+++								
	IgG3											
	IgG Buffer	+										

1

2 Binding of 11BD-2E11-2 to the above-mentioned panel of cancer and normal cell

3 lines plus the following additional ovarian cancer cell lines (A2780-cp, A2780-s, C-14,

4 OV2008, Hey, OCC-1, OVCA-429 and ES-2+SEAP) was assessed by flow cytometry

5 (FACS). Cells were prepared for FACS by initially washing the cell monolayer with

6 DPBS (without Ca⁺⁺ and Mg⁺⁺). Cell dissociation buffer (INVITROGEN, Burlington,

7 ON) was then used to dislodge the cells from their cell culture plates at 37°C. After

8 centrifugation and collection the cells were resuspended in Dulbecco's phosphate buffered

9 saline containing MgCl₂, CaCl₂ and 2 or 25 percent fetal bovine serum (FBS) at 4°C (wash

10 media) and counted, aliquoted to appropriate cell density, spun down to pellet the cells and

11 resuspended in staining media (DPBS containing MgCl₂ and CaCl₂ +/- 2 percent FBS)

12 containing 11BD-2E11-2 or control antibodies (isotype control or anti-EGFR) at 20 μ g/mL

13 on ice for 30 min. Prior to the addition of Alexa Fluor 488-conjugated secondary antibody

14 the cells were washed once with wash media. The Alexa Fluor 488-conjugated antibody in

15 staining media was then added for 20 to 30 min. The cells were then washed for the final

16 time and resuspended in staining media containing 1 μ g/mL propidium iodide or 1.5

17 percent paraformaldehyde. Flow cytometric acquisition of the cells was assessed by

Table 3		BREAST			COLON			LUNG	OVARY	PROSTATE	NORMAL		
Antibody	Isotype	MB-231	MB-468	MCF-7	HT-29	SW1116	SW620	NCI H460	OVCA8	PC-3	OCD 27sk	OCD-112	Hs888 Lu
11BD-2E11-2	IgG1, k	+	-	-	-	-	-	-	-	-	+	+	+
anti-EGFR	IgG1, k	++	++	-	+	+	-	+	+	+	+	+	+

1

Table 4		Ovarian							
Antibody	Isotype	A2780-cp	A2780-s	C-14	OV2008	ES-2+SEAP	Hey	OCC-1	OVCA-429
11BD-2E11-2	IgG1, k	+	+	-	-	+	+	+	-
anti-EGFR	IgG1, k	-	-	+	+	+	+	+	+

2

3 running samples on a FACScan using the CellQuest software (BD Biosciences, Oakville,
4 ON). The forward (FSC) and side scatter (SSC) of the cells were set by adjusting the
5 voltage and amplitude gains on the FSC and SSC detectors. The detectors for the three
6 fluorescence channels (FL1, FL2, and FL3) were adjusted by running cells stained with
7 purified isotype control antibody followed by Alexa Fluor 488-conjugated secondary
8 antibody such that cells had a uniform peak with a median fluorescent intensity of
9 approximately 1-5 units. Live cells were acquired by gating for FSC and propidium iodide
10 exclusion (when used). For each sample, approximately 10,000 live cells were acquired for
11 analysis and the results presented in Table 3 and 4. Tables 3 and 4 tabulated the mean
12 fluorescence intensity fold increase above isotype control and is presented qualitatively as:
13 less than 5 (-); 5 to 50 (+); 50 to 100 (++); above 100 (+++) and in parenthesis, the
14 percentage of cells stained.

15 Representative histograms of 11BD-2E11-2 antibodies were compiled for Figure 5.
16 11BD-2E11-2 displayed specific tumor binding to the breast tumor cell line MDA-MB-231
17 (Table 3) and several ovarian tumor cell lines including ES-2+SEAP (Table 4). There was
18 also binding of 11BD-2E11-2 to non-cancer cells, however that binding did not produce
19 cytotoxicity. This was further evidence that binding was not necessarily predictive of the

outcome of antibody ligation of its cognate antigen, and was a non-obvious finding. This suggested that the context of antibody ligation in different cells was determinative of cytotoxicity rather than just antibody binding.

Example 5

Normal Human Tissue Staining

IHC studies were conducted to characterize 11BD-2E11-2 antigen distribution in humans. IHC optimization studies were performed previously in order to determine the conditions for further experiments. 11BD-2E11-2 monoclonal antibody was produced and purified as stated above.

Binding of antibodies to 20 normal human tissues was performed using a frozen human normal organ tissue array (Clinomics, Watervliet, NY). Slides were postfixated for 10 min in cold (-20°C) acetone and then allowed to come to room temperature. Slides were rinsed in 4°C cold phosphate buffered saline (PBS) 3 times for 2 min each followed by blocking endogenous peroxidase activity with washing in 3 percent hydrogen peroxide for 10 min. Slides were then rinsed in PBS 3 times for 5 min followed by incubation in Universal blocking solution (Dako, Toronto, Ontario) for 5 min at room temperature. 11BD-2E11-2, anti-human muscle actin (Clone HHF35, Dako, Toronto, Ontario) or isotype control antibody (directed towards *Aspergillus niger* glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissues; Dako, Toronto, Ontario) were diluted in antibody dilution buffer (Dako, Toronto, Ontario) to its working concentration (5 µg/mL for each antibody except for anti-actin which was 2 µg/mL) and incubated overnight for 1 hr at room temperature. The slides were washed with PBS 3 times for 2 minutes each. Immunoreactivity of the primary antibodies was detected/visualized with HRP conjugated secondary antibodies as supplied (Dako Envision System, Toronto,

1 Ontario) for 30 min at room temperature. Following this step the slides were washed with
2 PBS 3 times for 2 min each and a color reaction developed by adding DAB (3,3'-
3 diaminobenzidine tetrahydrachloride, Dako, Toronto, Ontario) chromogen substrate
4 solution for immunoperoxidase staining for 10 min at room temperature. Washing the
5 slides in tap water terminated the chromogenic reaction. Following counterstaining with
6 Meyer's Hematoxylin (Sigma Diagnostics, Oakville, ON), the slides were dehydrated with
7 graded ethanols (95-100%) and cleared with xylene. Using mounting media (Dako
8 Faramount, Toronto, Ontario) the slides were coverslipped. Slides were microscopically
9 examined using an Axiovert 200 (Zeiss Canada, Toronto, ON) and digital images acquired
10 and stored using Northern Eclipse Imaging Software (Mississauga, ON). Results were
11 read, scored and interpreted by a pathologist.

12 Table 5 presents a summary of the results of 11BD-2E11-2 staining of an array of
13 normal human tissues. From the table, there were 2 main categories of tissue staining. A
14 group of tissues was completely negative. These tissues included normal thyroid,
15 bronchus and cardiac muscle of the left ventricle (Figure 6). The second group of tissues
16 included tissues in which staining was positive in the tissue section, but was limited to
17 smooth muscle fibers of blood vessels and/or the epithelium (Figure 7). These results
18 suggested that the antigen for 11BD-2E11-2 was not widely expressed on normal tissues,
19 and that the antibody would bind only to a limited number of tissues in humans. The
20 normal human tissue staining of 11BD-2E11-2 resembles that previously reported for an
21 anti-MCSP antibody; B5. B5 was previously shown to bind to skin keratinocytes, lung
22 alveolar epithelium and capillary endothelium.

1	Bronchus	61	M	- (PD)	+++ SMF & Myoepithelium of mucus acini	CD
2	Diaphragm	61	M	+++ SMF of blood vessels +/- Skeletal muscle fibers	+++ Skeletal muscle fibers & SMF of blood vessels	-
3	Pectoral muscle (Skeletal muscle)	61	M	+++ SMF of blood vessels	+++ Skeletal muscle fibers & SMF of blood vessels	-
4	Lung	61	M	+++ Alveolar epithelium & SMF of blood vessels	CD	- (F)
5	Aorta	61	M	++ SMF (F)	CD	-
6	Left ventricle (Cardiac muscle)	61	M	-	+++ SMF of blood vessels + Cardiac muscle fibers	-
7	Esophagus	61	M	+++ SMF (PD)	CD	- (F)
8	Trachea	61	M	- (PD)	+++ SMF & myoepithelium of mucus acini	-
9	Kidney	61	M	+++ SMF of blood vessels	+++ SMF of blood vessels	-
10	Adrenal	61	M	+++ SMF of blood vessels	+++ SMF of blood vessels	-
11	Pancreas	61	M	+++ SMF of blood vessels + Acinar epithelium	+++ SMF of blood vessels	-
12	Spleen	61	M	+++ SMF of blood vessels & Polymorphs (F)	+++ SMF of blood vessels, reticular fibers & polymorphs (F)	Bg (polymorphs)
13	Liver	61	M	+++ SMF of blood vessels	- (PD)	-
14	Skin	61	M	+++ SMF of blood vessels +/- Keratinocytes	+++ SMF of blood vessels	Bg (Stroma)
15	Colon	61	M	+++ SMF of blood vessels	+++ SMF	-
16	Thyroid	61	M	- (PD)	- (PD)	-
17	Prostate	61	M	++ SMF of blood vessels +/- Glandular epithelium	CD	CD
18	Testicle	61	M	++ SMF of blood vessels	+++ stromal cells	-
19	Breast	61	M	+/- Ductal epithelium +++ SMF of blood vessels	+++ SMF of blood vessels	-
20	Ovary	80	F	++ SMF of blood vessels & Stroma	F	CD

Table 5: 11BD-2E11-2 IHC on Frozen Human Normal Tissue

Abbreviations: SMF: smooth muscle fiber, Bg: background staining, PD: partially detached, F: folded, CD: completely detached.

Example 6

Human Breast Tumor Tissue Staining

An IHC study was undertaken to determine the cancer association of the 11BD-2E11-2 antigen with human breast cancers. A comparison was made for actin (positive control), and an antibody directed towards *Aspergillus niger* glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissues (negative control). A breast cancer tissue array derived from 15 breast cancer patients and 5 samples derived from non-neoplastic breast tissue in breast cancer patients were used (Clinomics, Watervliet, NY). The following information was provided for each patient: age, sex, and diagnosis. The procedure for IHC from Example 5 was followed.

Table 6 provides a binding summary of 11BD-2E11-2 antibody staining of a breast cancer tissue array. Each array contained tumor samples from 15 individual patients. Overall, 62 percent of the 8 (7 of the tissue samples were either completely detached or not

1 representative) patients tested were positive for the 11BD-2E11-2 antigen. Also for 11BD-
2 2E11-2, 0 out of 3 (again 2 of the tissue samples were completely detached) normal breast
3 tissue samples from breast cancer patients were positive (Figure 8). For the 11BD-2E11-2
4 antigen there did not appear to be a trend to greater positive expression with higher tumor
5 stage. However, this result was limited due to the small sample size. The 11BD-2E11-2
6 staining was specific for cancerous cells (Figure 8). The staining pattern, from 11BD-
7 2E11-2, showed that in patient samples, the antibody was highly specific for malignant
8 cells thereby making it an attractive druggable target. The breast tumor tissue staining of
9 11BD-2E11-2 resembles that previously reported for the anti-MCSP antibody B5. B5 was
10 previously shown to bind to 60 percent of breast carcinoma tumor tissue.

Data Sheet					IHC Score		
S. NO.	Tissue	Age	Sex	Diagnosis	11BD-2E11-2	Actin	IgG negative control
1	Breast	61	F	Infiltrating Ductal Carcinoma	CD	CD	CD
2	Breast	74	F	Infiltrating Ductal Carcinoma	- (PD)	- Tumor +++ SMF of blood vessels	-
3	Breast	60	F	Infiltrating Ductal Carcinoma	CD	PD	CD
4	Breast	69	F	Infiltrating Ductal Carcinoma	NR	NR	-
5	Breast	64	F	Infiltrating Ductal Carcinoma	CD	-	CD
6	Breast	65	F	Medullary Carcinoma	+++ (Tumor cells)	-	-
7	Breast	75	F	Infiltrating Ductal Carcinoma	+++ (Tumor cells)	CD	-
8	Breast	48	F	Infiltrating Ductal Carcinoma	++ (Tumor cells)	- Tumor ++ Stroma	-
9	Breast	87	F	Infiltrating Ductal Carcinoma	+/- (Tumor cells)	- Tumor +++ SMF of blood vessels	CD
10	Breast	75	F	Infiltrating Ductal Carcinoma	NR (+/- SMF of blood vessels)	CD	-
11	Breast	76	F	Infiltrating Ductal Carcinoma	-	- Tumor +++ SMF of blood vessels & stroma	-
12	Breast	66	F	Infiltrating Ductal Carcinoma	CD	CD	-
13	Breast	58	F	Infiltrating Ductal Carcinoma	+++ (Tumor cells)	CD	CD
14	Breast	37	F	Infiltrating Ductal Carcinoma	CD	- Tumor +++ Stroma	-
15	Breast	70	F	Infiltrating Ductal Carcinoma	-	- Tumor +++ Myoepithelium & SMF of blood vessels	CD
16	Breast	48	F	Normal	- (PD)	CD	CD
17	Breast	60	F	Normal	-	- (PD)	-
18	Breast	30	F	Normal	CD	- Tumor +++ Myoepithelium & SMF of blood vessels	
19	Breast	34	F	Normal	CD	- Tumor ++ Myoepithelium (PD)	
20	Breast	43	F	Normal	-	- Tumor + SMF of blood vessels	

11 Table 6: 11BD-2E11-2 IHC on Frozen Human Normal and Breast Tumor Tissue

12 Abbreviations: SMF: smooth muscle fiber, PD: partially detached, F: folded, CD:
13 completely detached.

1
2

3 Example 7

4 *In Vivo* MDA-MB-468 Established Tumor Experiment

5 With reference to Figure 9, 6 to 8 week old female SCID mice were implanted with
6 2 million MDA-MB-468 human breast cancer cells in 100 microlitres saline injected
7 subcutaneously in the scruff of the neck. Tumor growth was measured with calipers every
8 week. When the majority of the cohort reached a tumor volume of 100 mm³, 5-6 mice were
9 randomized into each of 2 treatment groups. 11BD-2E11-2 or buffer control was
10 administered intraperitoneally with 10 mg/kg/dose at a volume of 300 microliters after
11 dilution from the stock concentration with a diluent that contained 2.7 mM KCl, 1 mM
12 KH₂PO₄, 137 mM NaCl and 20 mM Na₂HPO₄. The antibodies were then administered 3
13 times per week for a total of 10 doses in the same fashion until day 66 post-implantation.
14 Tumor growth was measured about every seventh day with calipers for the duration of the
15 study or until individual animals reached CCAC end-points. Body weights of the animals
16 were recorded for the duration of the study. At the end of the study all animals were
17 euthanised according to CCAC guidelines.

18 At the time of randomization the mean tumor volumes and the standard deviations
19 in each group were similar. Statistically there was no difference in body weight between
20 the groups. This indicated that true randomization had occurred. As shown in Figure 9, the
21 antibody 11BD-2E11-2 suppressed tumor growth by 25 percent in comparison to buffer
22 control at the end of the 3-week treatment period (p=0.52). Although this was not a
23 significant difference, a trend towards reduced tumor volume in comparison to the buffer

control was observed throughout the study. Therefore, 11BD-2E11-2 has shown efficacy in an established breast cancer model.

Example 8

In Vivo ES-2+SEAP Established Tumor Experiment

With reference to Figures 10 and 11, 6 to 8 week old female athymic nude mice were intraperitoneally implanted with 10 million ES-2+SEAP human ovarian cancer cells stably transfected to express human placental secreted alkaline phosphatase (SEAP). The 10 million ovarian cancer cells were resuspended in 500 microlitres serum-free α -MEM. Tumor growth was confirmed with the sacrifice of 3 mice on day 7. Following the confirmation of tumor growth on day 7, 8 mice were randomized into each of 2 treatment groups. 11BD-2E11-2 or buffer control was administered intraperitoneally with 10 mg/kg/dose at a volume of 250 microliters after dilution from the stock concentration with a diluent that contained 2.7 mM KCl, 1 mM KH_2PO_4 , 137 mM NaCl and 20 mM Na_2HPO_4 . The antibodies were then administered once per day for 5 doses and then once every other day for another 5 doses for a total of 10 doses. Tumor burden was extrapolated by measuring circulating SEAP levels and assessed visually upon necropsy for the duration of the study or until individual animals reached CCAC end-points. Body weights of the animals were recorded for the duration of the study. At the end of the study all animals were euthanised according to CCAC guidelines.

At the time of randomization circulating plasma SEAP levels (indicative of tumor burden) were analyzed. There was not a significant difference in the average SEAP level between the 11BD-2E11-2 and buffer control treatment group. However, within groups there was variable tumor take-rate. As shown in Figure 10, the antibody 11BD-2E11-2 displayed a trend for improved survival in a cohort of the treatment group. As illustrated

1 in Figure 11, one animal receiving 11BD-2E11-2 treatment had a decreased amount of
2 circulating SEAP to nearly negligible levels. The low level of circulating SEAP continued
3 on until approximately 60 days post-implantation. In all, these results in which 11BD-
4 2E11-2 produced benefits (improved survival and decreased tumor burden in comparison
5 to control treatment) in multiple models of human cancer suggest pharmacologic and
6 pharmaceutical benefits of this antibody for cancer therapy in mammals, including man.

7 The preponderance of evidence shows that 11BD-2E11-2 mediates anti-cancer
8 effects through ligation of an epitope present on MSCP. For the purpose of this invention,
9 said epitope is defined as a "MSCP antigenic moiety" characterized by its ability to bind
10 with a monoclonal antibody encoded by the hybridoma cell line 11BD-2E11-2, antigenic
11 binding fragments thereof or antibody conjugates thereof. It has been shown, in Example
12 3, 11BD-2E11-2 antibody can be used to immunoprecipitate the cognate antigen from
13 expressing cells such as MDA-MB-231 cells. Further it could be shown that the 11BD-
14 2E11-2 antibody could be used in detection of cells and/or tissues which express a MSCP
15 antigenic moiety which specifically binds thereto, utilizing techniques illustrated by, but
16 not limited to FACS, cell ELISA or IHC.

17 Thus, it could be shown that the immunoprecipitated 11BD-2E11-2 antigen can
18 inhibit the binding of 11BD-2E11-2 to such cells or tissues using FACS, cell ELISA or
19 IHC assays. Further, as with the 11BD-2E11-2 antibody, other anti-MSCP antibodies
20 could be used to immunoprecipitate and isolate other forms of the MSCP antigen, and the
21 antigen can also be used to inhibit the binding of those antibodies to the cells or tissues that
22 express the antigen using the same types of assays.

23 All patents and publications mentioned in this specification are indicative of the
24 levels of those skilled in the art to which the invention pertains. All patents and

1 publications are herein incorporated by reference to the same extent as if each individual
2 publication was specifically and individually indicated to be incorporated by reference.

3 It is to be understood that while a certain form of the invention is illustrated, it is
4 not to be limited to the specific form or arrangement of parts herein described and shown.
5 It will be apparent to those skilled in the art that various changes may be made without
6 departing from the scope of the invention and the invention is not to be considered limited
7 to what is shown and described in the specification. One skilled in the art will readily
8 appreciate that the present invention is well adapted to carry out the objects and obtain the
9 ends and advantages mentioned, as well as those inherent therein. Any oligonucleotides,
10 peptides, polypeptides, biologically related compounds, methods, procedures and
11 techniques described herein are presently representative of the preferred embodiments, are
12 intended to be exemplary and are not intended as limitations on the scope. Changes therein
13 and other uses will occur to those skilled in the art which are encompassed within the spirit
14 of the invention and are defined by the scope of the appended claims. Although the
15 invention has been described in connection with specific preferred embodiments, it should
16 be understood that the invention as claimed should not be unduly limited to such specific
17 embodiments. Indeed, various modifications of the described modes for carrying out the
18 invention which are obvious to those skilled in the art are intended to be within the scope
19 of the following claims.

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